

Lethal and Sublethal Effects of a Simulated Salt Brine Effluent on Adults and Subadults of the Shrimps *Penaeus setiferus* and *P. aztecus*

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Abstract

Lethal and certain sublethal effects of salt brines on adults and subadults of two species of penaeid shrimps, *Penaeus setiferus* and *P. aztecus*, were examined to evaluate the potential impact of ocean disposal of brine from solution mining of salt domes. Brines, prepared from dome salt or synthetic sea salt diluted with Brazos River (Texas, USA) water or deionized water, were mixed with seawater and delivered from a proportional diluter to shrimp held (usually) at 25 °C. For each combination of species, salt, and diluent, 90-individual trials were conducted in the fall and spring. The effects of temperature were evaluated separately. Median lethal time was strongly dose-dependent. Median lethal concentrations at 48 and 96 h were 654 ± 42 (95% confidence interval) and 540 ± 41 mOsm kg⁻¹ above ambient seawater, respectively, well above the worst-case predictions for the brine-disposal area. Salt type, diluent type, season or species did not significantly affect brine lethality. Mortality was higher for both species at 30 °C and lower for *P. setiferus* and higher for *P. aztecus* below 25 °C. Lethal brine doses produced tachycardia after 6 (*P. setiferus*) or 12 h (*P. aztecus*) of brine exposure. Opacity of abdominal muscles increased with brine concentration. Lethal brine concentrations evoked hyperactivity after 0.75–1.5 h of exposure, significant failure to orient after 6 h and a reduction in general activity after 12 h. Behavior and osmoregulation suggest higher sensitivities to brines made with dome salt or river water and in shrimp tested during the cool seasons.

Introduction

In March, 1980, the US Department of Energy (DOE) began to discharge brine, consisting of salt from the Bryan Mound salt dome dissolved in Brazos River water, at rates exceeding 100 000 barrels d⁻¹ from a diffuser located 20 km offshore from Freeport, Texas. One recurrent public concern has been that the brine discharge may adversely affect the commercial penaeid shrimp fishery, whether by direct toxic effects or by physiological or behavioral perturbations. Information of several kinds must be integrated to adequately assess those potentially adverse impacts:

- (1) Hydrographic profiles of the potentially affected area.
- (2) Estimates of the brine plume's behavior in space and time under a suitable range of environmental parameters.
- (3) Natural history of penaeid shrimp in the potentially affected area.
- (4) Toxic and sublethal effects of brine and its components on penaeid shrimp.

Reasonably good information is presently available for the first three items (NOAA, 1977; Metzbower *et al.*, 1980). An addition to our understanding of the effects of brine was the goal of the present study.

The brine diffuser is located in water approximately 20 m deep at the bottom of a water column characterized by considerable vertical stability (Metzbower *et al.*, 1980). In this report, bottom salinities measured for 1 yr at the sampling station nearest the diffuser site or at depths greater than 15 m near the diffuser averaged slightly more than 34‰ S (range 32.4–37.0‰ S). Since the high density of the brine should confine its major effects to within 2 m of the bottom (NOAA, 1978), it is reasonable to assume that any effects of brine will occur in water with a normal ambient salinity of 34‰ S. Temperatures of water deeper than 15 m near the site ranged from a summer high of 30 °C to a winter low of 10 °C.

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Brine plume behavior has been modeled for a variety of projected current regimes (NOAA, 1977). Near field (0–30 m) effects are considered to be dominated by diffuser jet mixing and, based on a worst-case estimate of 1:45 dilution, should increase salinity by about 6.5‰ S in the presence of little or no current. The intermediate field (30–300 m) is dominated by lateral spreading of the brine. Beyond 300 m dilution is produced by advection. The transient plume model has been used (NOAA, 1978) to simulate expected durations of Eulerian (fixed with respect to the sea floor) exposure to various levels of increased salinity at selected locations near the diffuser for a simulated 90-d discharge. That simulation indicates that locations 600 m parallel to the shore from the diffuser should be exposed to an increased salinity of 2‰ S for no more than 24 h at a time. Though penaeid shrimp are the dominant invertebrate nekton species in the diffuser area (Metzbower *et al.*, 1980), it is likely that most penaeid shrimp spawn well inshore (white shrimp) or offshore (brown shrimp) from the expected far field limits (Galloway and Reitsema, 1981).

Whether brine at the projected concentrations will kill or disturb large penaeid shrimp in the area has not been explored, though some inferences can be drawn from related studies. Potential impact from brine consists of two components, elevated salinity and possible river water contamination. Further, the mode of action for elevated salinity is far from clear. As Neff *et al.* (1979) pointed out, salt dome salt may be toxic because of toxic trace metals, because of altered major ion ratios (especially $\text{Ca}^{++}/\text{Mg}^{++}$), or because it imposes an additional osmotic stress. Few studies have considered more than the last of these possibilities. The most comprehensive and applicable study of salinity effects on larger individuals of either species in question is that of Venkataramiah *et al.* (1974). Those results based on static bioassays suggest that the expected increase in salinity near the diffuser is well within the tolerance limits of the juvenile brown shrimp *Penaeus aztecus* but that tolerance to extremes of salinity may decrease with increasing size up to 75 mm (=4 cm tail length). Larger individuals were not examined. The toxicity of a chemical feedstock (Dow Chemical) derived from Bryan Mound salt for early stages of the white shrimp *P. setiferus*, has also been examined (Wilson *et al.*, 1979). Those results, though rather difficult to interpret, suggest that lethal limits for postlarvae may be as low as 38‰ S (LD50-96) and that brine tolerance limits may decrease with increasing temperature above 28 °C.

The present study examines lethal and certain sublethal effects of a diluted, simulated brine effluent and of a series of three control brines on adults and subadults of *Penaeus setiferus* and *P. aztecus*.

Materials and Methods

Test Individuals

Individuals of *Penaeus setiferus* and *P. aztecus* from 6–11 cm in tail length (posterior carapace margin to tip of

telson) were captured by commercial shrimp trawl offshore from Freeport, Texas, then maintained in a 15 000-l outdoor holding tank equipped with sand substratum and subgravel filters at the laboratory in Galveston, Texas. Seawater was continually replaced in the holding tank at 0.3 tank volumes d^{-1} , and shrimp were fed approximately 20% of their volume of chopped, frozen squid daily during holding. Individuals were held for at least 5 d before experiments. Mortality, salinity and temperature in the holding tank were monitored daily. Holding tank temperatures ranged from 15° to 31 °C during the experiments but never differed from temperatures at the site of capture by more than 2 °C.

Acute Toxicity Experiments

For toxicity bioassays shrimp were transferred to glass, 40-l aquaria subdivided with plastic screen to accommodate 5 individuals each. Tanks were placed in a constant-environment room and fitted with constant-level siphons adjusted to maintain water volumes of 25 l. Aeration was provided in each chamber by a full-length, perforated-plastic air diffuser in each tank. Individuals were exposed to 650 lx of fluorescent illumination for 14 h daily, preceded and followed by 30 min of 200 lx simulated dawn and dusk. Air temperature was held at $25 \pm 0.1 \text{ °C}$ with a seawater heat exchanger (Neslab SWHX + PBC-2). Temperatures were continuously monitored in room air and in 5 of 18 test tanks.

Four test brines were mixed 800 l at a time by adding either Instant Ocean Synthetic Sea Salts without trace element mix (Aquarium Systems, Inc.) or Ranch House Coarse Salt (United Salt Co.) at approximately 320 g l^{-1} to deionized, charcoal-filtered water ($< 1 \mu\text{mho cm}^{-1}$) or to water collected in plastic carboys from the Brazos River at the intake structure for the DOE solution mining program. Instant Ocean was selected as a control salt to test potential effects of Brazos River water in brine, and Ranch House Salt, mined from a salt dome at Hockley, Texas, was selected as a substitute for Bryan Mound salt which was unavailable in crystalline form. Both salts, both diluents and a sample of natural seawater from the laboratory's supply were analyzed for major ions and certain trace elements by a commercial laboratory (Table 1). This analysis confirmed the similarity in major ion concentrations between salts from the Hockley and Bryan Mound domes. Copper and cadmium concentrations in seawater were measured by Dr. P. Boothe (Texas A&M University), using flameless atomic absorption spectrometry after coprecipitation (Fe) with APDC. Salt and diluent suspensions were continually agitated (and heated to 35 °C during cold weather) until the specific gravity of the clear supernatant liquid reached 1.20, at which time the supernatant brine was pumped into reservoirs.

Brine flowed by gravity into a head tank in the aquarium room from which it was diluted with seawater in a modified proportional diluter (Mount and Brungs, 1967).

Table 1. Chemical analyses of test salts, test diluents, and laboratory seawater

Ion	Unit	Deionized water	Brazos River water ^a	Seawater	Ranch House salt	Instant Ocean	Bryan Mound ^b
Na ⁺	ppm	17	844 (4 000)	9 300	461 000	414 000	373 800
Cl ⁻	ppm	<5	4 780 (6 200)	19 990	617 300	537 000	617 000
K ⁺	ppm	3	190 (200)	750	5 600	18 100	940
Mg ⁺⁺	ppm	5	247 (380)	958	30	82 500	29
Ca ⁺⁺	ppm	1	55 (200)	325	9 750	16 000	2 290
SO ₄ ⁻⁻	ppm	12	576 (890)	2 100	23 650	56 600	6 230
Cu ⁺⁺	ppb	< 10	12	1.05 ^c	< 10	< 10	< 30
Cd ⁺⁺	ppb	< 50	< 50	0.085 ^c	< 50	< 50	< 6
Zn ⁺⁺	ppb	< 10	< 10	254
Ag ⁺	ppb	< 10	< 10	< 30

^a Values in parentheses provided by Neff, personal communication

^b Values for Bryan Mound salt are recalculated from measurements of a 317‰ S brine reported by Texas A&M University (James, 1977)

^c Measured by Dr. Paul Boothe, Dept. of Oceanography, Texas A&M University

Modifications included elimination of the toxicant-mixing cell to accommodate proportions of brine up to 10%, replacement of the standard solenoid (or needle) water and toxicant valves with a mechanical tubing valve that functioned well with saturated salt brine, and construction with acrylic sheet instead of glass. Outputs from each of five dosing chambers and the single control chamber were divided into three equal streams (Benoit and Puglisi, 1973) that were passed into the three test tanks for each dose, yielding 18 test tanks, containing a total of 90 individuals for each test. The diluter was adjusted to deliver five tank volumes (125 l) per day to each tank.

Individuals without obvious lesions or missing appendages were placed in the chambers of the test tanks that had been filled with water at holding tank temperature. Chamber assignments were randomized by lots. Test tank temperature was then gradually brought to 25 °C over 24 h, if holding tank temperature was within 5 °C of 25 °C, or over 48 h, if holding tank temperature was 15°–20 °C. Shrimp were not fed during this acclimation period or during the subsequent bioassay.

A dose series was selected in a range-finding static bioassay. For each experiment brine concentrations ranged from approximately 40 to 50‰ S, with adjacent doses differing by a factor of 1.17 in total osmolality. Since brines prepared from synthetic sea salts and salt dome salt differed in the relationship between salinity (as g l⁻¹) and osmolality (as mOsm kg⁻¹), brine doses were expressed as mOsm kg⁻¹, above control seawater osmolality, and osmolalities of all brine seawater solutions were measured with a vapor-pressure osmometer (Wescor 5100B).

At the conclusion of the 24- or 48-h acclimation period (10:00 to 12:00 hrs local time), toxicity experiments were

begun by turning on brine flow to the proportional diluter. This method of initiating brine delivery provided gradual exposure of test individuals to final toxicant concentration. At five exchanges per day, salt concentration reached 75% of its final value within about 7 h and 90% after about 12 h. Observations were made at brine flow initiation and 0.75, 1.5, 3, 6, 12, 24, 48, 72 and 96 h later. The use of this modified logarithmic time series permitted estimates of mortality proportion at fixed times and of mean time to death as a function of dose (Sprague, 1969).

Visual observations included mortality, molting, behavior and transparency for all shrimp and heart beat rate (time for 10 beats) for five quiescent individuals at each dose. Death was defined as lack of movement, heart beat and scaphognathite (gill bailer) movement, with failure to move in response to repeated touches with a probe. Behavior was scored as quiescent for individuals oriented normally on the tank bottom or clinging to a chamber divider without pleopod or periopod movement, active for swimming shrimp or individuals that oriented normally and showed pleopod movement, and moribund for live individuals resting on the tank bottom without the dorsal surface uppermost. Transparency was scored on a subjective scale from 0 (translucent) to 4 (very opaque muscles with marked cuticular mottling). Dead specimens were removed, measured and sexed, and exuvia were removed at each observation. After the 96-h observation, 5 individuals from the survivors in each dose (fewer, if fewer than 5 survived) were selected by lots, then blotted for 30 s, weighed, measured and sexed. The cuticle over the pericardium was then removed, the pericardium slit and hemolymph (about 5 µl) transferred to a paper disc to determine hemolymph osmolality. Carcasses were then

dried to constant weight at 90°C and reweighed to determine body water content.

Median lethal time (LT50) was computed for doses in which significant mortality occurred by graphic approximation to probit analysis (Litchfield and Wilcoxon, 1949). Median lethal brine concentration (LC50) was computed at 48 and 96 h by the trimmed Spearman-Kärber method (Hamilton *et al.*, 1977). Analyses of variance were performed with program P2V of the BMDP series (UCLA, 1977). Experiments were performed twice for each combination of species, salt type, and diluent type, once during the period from April through August (spring) and once from September through December (fall). In addition, the effects of temperature on brine toxicity were investigated in a series of shorter (48 h) experiments at 15°, 20°, and 30°C performed with Brazos River water and dome salt with two doses plus control and 10 shrimp per dose.

Results

Acute Brine Toxicity

Each of the 16 experiments furnished from 1–5 estimated of LT50, depending on the rate of mortality caused by the 5 doses in each experiment. Log (LT50) was strongly negatively correlated with log (dose) for both *Penaeus setiferus* ($r = -0.81$, $P < 0.01$) and *P. aztecus* ($r = -0.81$, $P < 0.01$) (Fig. 1). Species regression lines did not differ

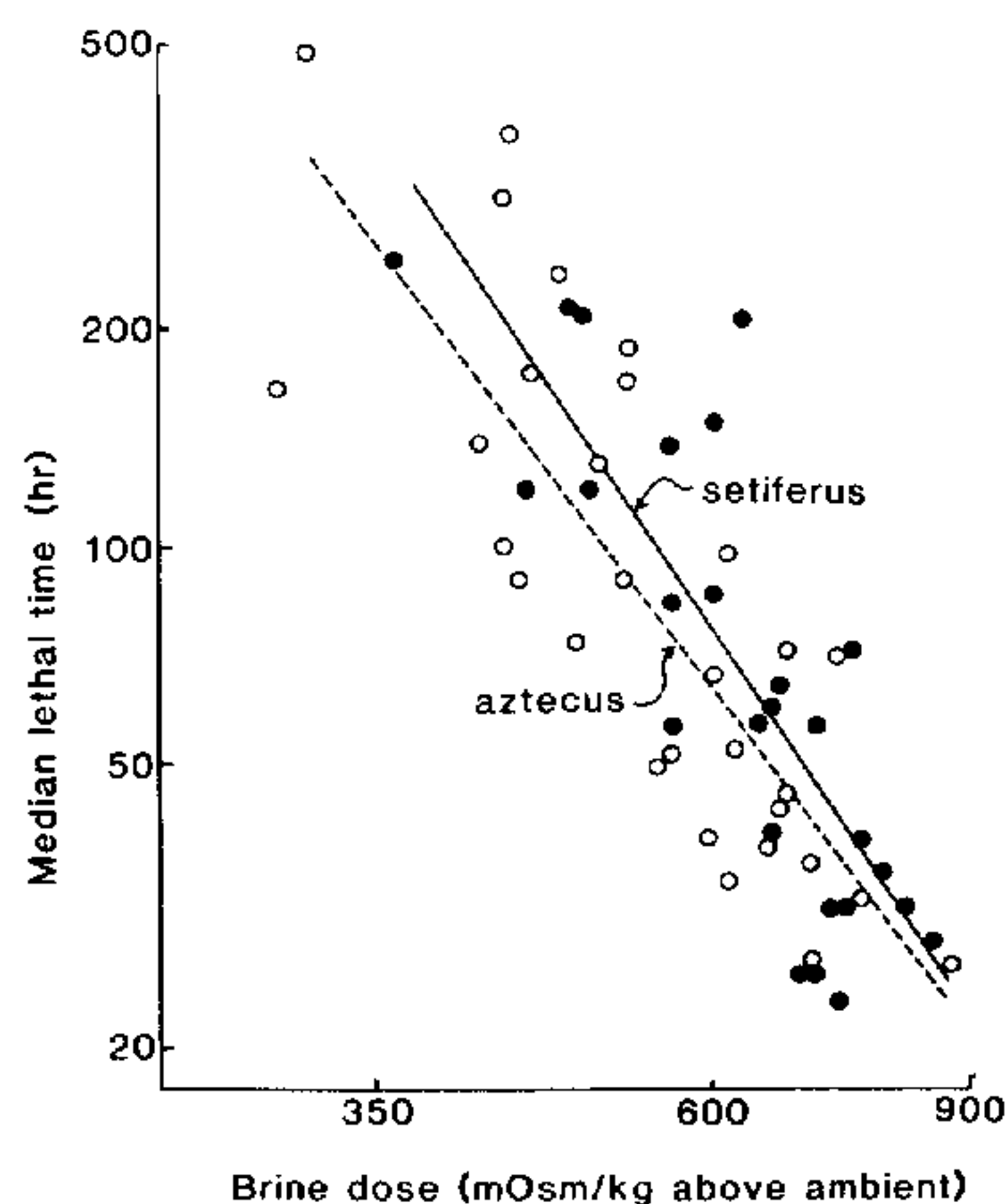


Fig. 1. *Penaeus setiferus* and *P. aztecus*. Median lethal time as a function of dose for *P. setiferus* (closed circles) and *P. aztecus* (open circles) with regression lines for each species. Both axes are logarithmic

significantly ($P > 0.5$). The design of the test (96 h duration with gradual brine onset) did not allow meaningful LT50 estimates of less than 20 h or more than about 400 h. LT50 values were subjected to analysis of variance to detect potential effects of species, salt type, diluent type, or shrimp size (log length) on LT50 adjusted for covariance

Table 2. Means of median lethal time (LT50 in h) estimates for each experiment, adjusted for covariance with dose

Species	Instant Ocean				Salt Dome			
	Deionized water		River water		Deionized water		River water	
	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
<i>P. setiferus</i>	149	94	120	92	95	135	105	94
<i>P. aztecus</i>	14	124	108	6	70	134	17	120

Analysis of variance Source	Degrees of freedom	Mean square	F ^a
Species	1	4 706	1.56
Salt	1	669	0.22
Diluent	1	2 126	0.71
Season	1	2 929	0.97
Species × salt	1	2 017	0.67
Species × diluent	1	117	0.04
Salt × diluent	1	311	0.10
Species × season	1	3 124	1.04
Salt × season	1	14 103	4.68 ^a
Diluent × season	1	5 435	1.81
First covariate (log Δ osmolality)	1	202 913	75.16 ^a
Second covariate (log length)	1	1 157	0.43
All covariates	1	116 922	43.31
Error	48	3 009	

^a F statistics indicating significant sources of variance, $P < 0.05$

Table 3. Control osmolalities and median lethal concentrations (mOsm kg⁻¹ above control) at 48 and 96 h for each experiment

Species	Instant Ocean				Salt Dome			
	Deionized water		River water		Deionized water		River water	
	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
<i>P. setiferus</i>								
Control	746	1 088	754	812	745	629	713	644
LC50-48	641	779	726	741 ^a	679	582	712	586 ^b
LC50-96	615	432	656	657	563	551	597	465 ^b
<i>P. aztecus</i>								
Control	823	1 007	732	1 036	790	615	807	641
LC50-48	541	659 ^a	794 ^b	625	634	593	532	637
LC50-96	439	518	632	460	555	506	462	533

^a estimate from probit plot^b estimate from LT50 regression

with log dose. Log length was used to correct skew in length measurements and to provide a parameter linearly related to log weight, since the weight was unobtainable for dead specimens. That analysis (Table 2) revealed no significant main effects of species, salt type, diluent type or season, no significant covariance of LT50 with shrimp size, and, as has been described above (Fig. 1), a highly significant covariance of log dose with LT50. The only other significant variance term, the salt type-season interaction term is the result of three anomalously low LT50 experimental means (adjusted for log dose covariance) for which there is no ready explanation.

Median lethal concentrations at 48 (LC50-48) and 96 h (LC50-96) could be calculated by the trimmed Spearman-Kärber method in all cases except five. For the exceptions,

excessive or insufficient mortality resulting from misaligned dose series required estimation of LC50 by extrapolation from log dose-probability plots or by extrapolation to 48 and 96 h from LT50 – log dose plots. Those estimates are identified in Table 3 with letters to show the estimation method used. Since control seawater osmolality ranged from a low of 615 mOsm kg⁻¹ to a high of 1088 mOsm kg⁻¹, it is to be expected that LC50, expressed as absolute osmolality, varied in response to control osmolality. Least squares regression indicated that LC50-96 rose at the rate of approximately 0.8 mOsm kg⁻¹ for each mOsm kg⁻¹ increase in acclimation osmolality (Fig. 2). That correspondence justified the expression of LC50 as the difference between control and lethal osmolalities.

LC50-48 and -96 were examined for effects of species, salt type, diluent type and season by analysis of variance (Model I, full factorial design). That analysis showed no significant variance contribution by any main effect or interaction between and among main effects. Grand means across all treatments gave best estimates for median lethal concentration of 654±42 (95% Confidence Interval) mOsm kg⁻¹ at 48 h and 540±41 mOsm kg⁻¹ at 96 h, values that correspond to salinity increases of approximately 22 and 18‰ S, respectively.

In general, sensitivity to salt dome-river water brine increased at 30 °C in both species, and *Penaeus setiferus* survived high salinity better as temperature was reduced to 15 °C (Fig. 3).

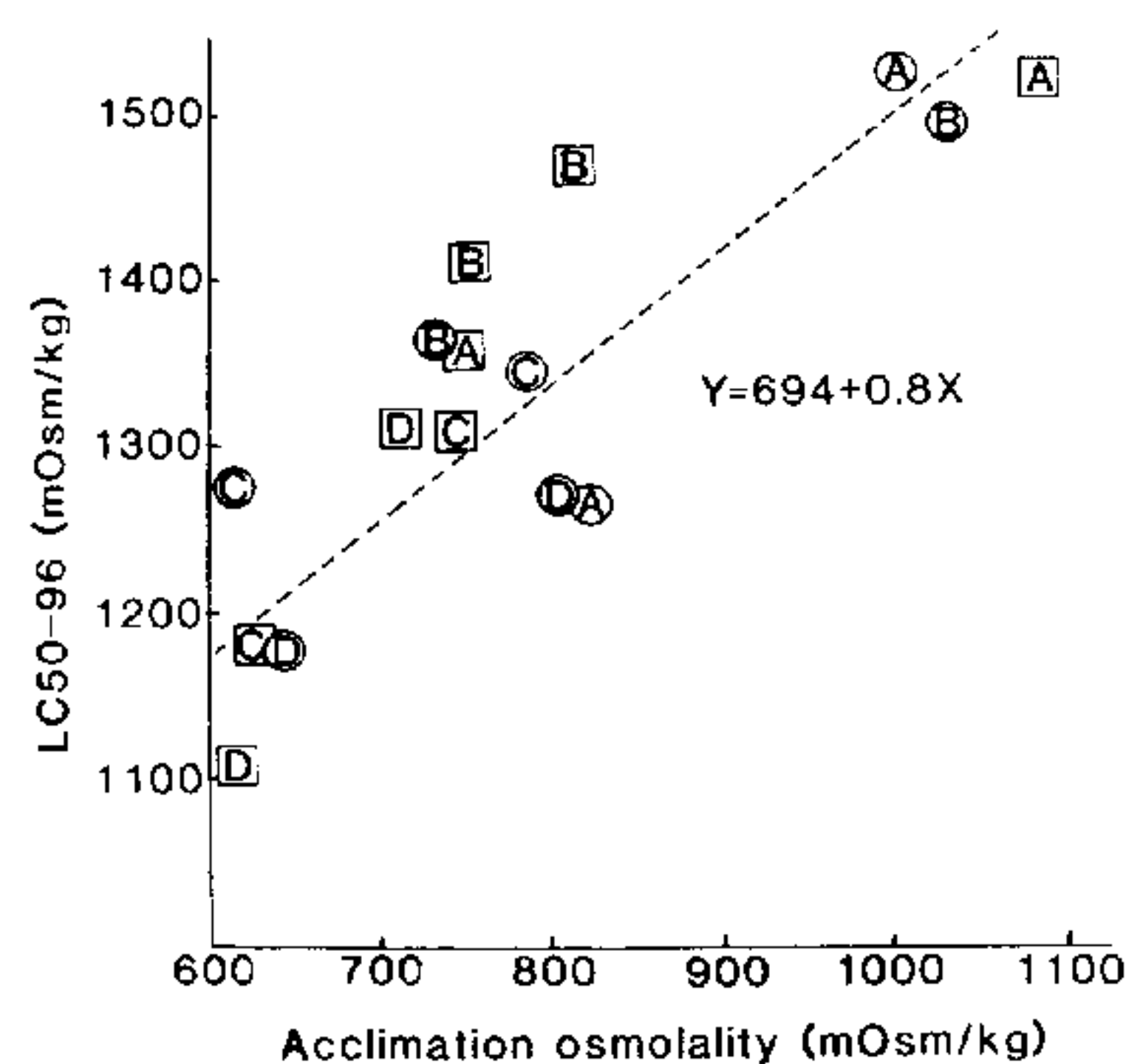


Fig. 2. *Penaeus setiferus* and *P. aztecus*. Median lethal concentration at 96 h as a function of control osmolality. Letters label treatments as follows: A = Instant Ocean/deionized water, B = Instant Ocean/river water, C = dome salt/deionized water, and D = dome salt/river water. Squares denote *P. setiferus* and circles *P. aztecus*

Sublethal Effects

Shrimp transparency scores and heart rates provided early evidence of brine stress in acute experiments. Transparency scores were subjective and variable with approxi-

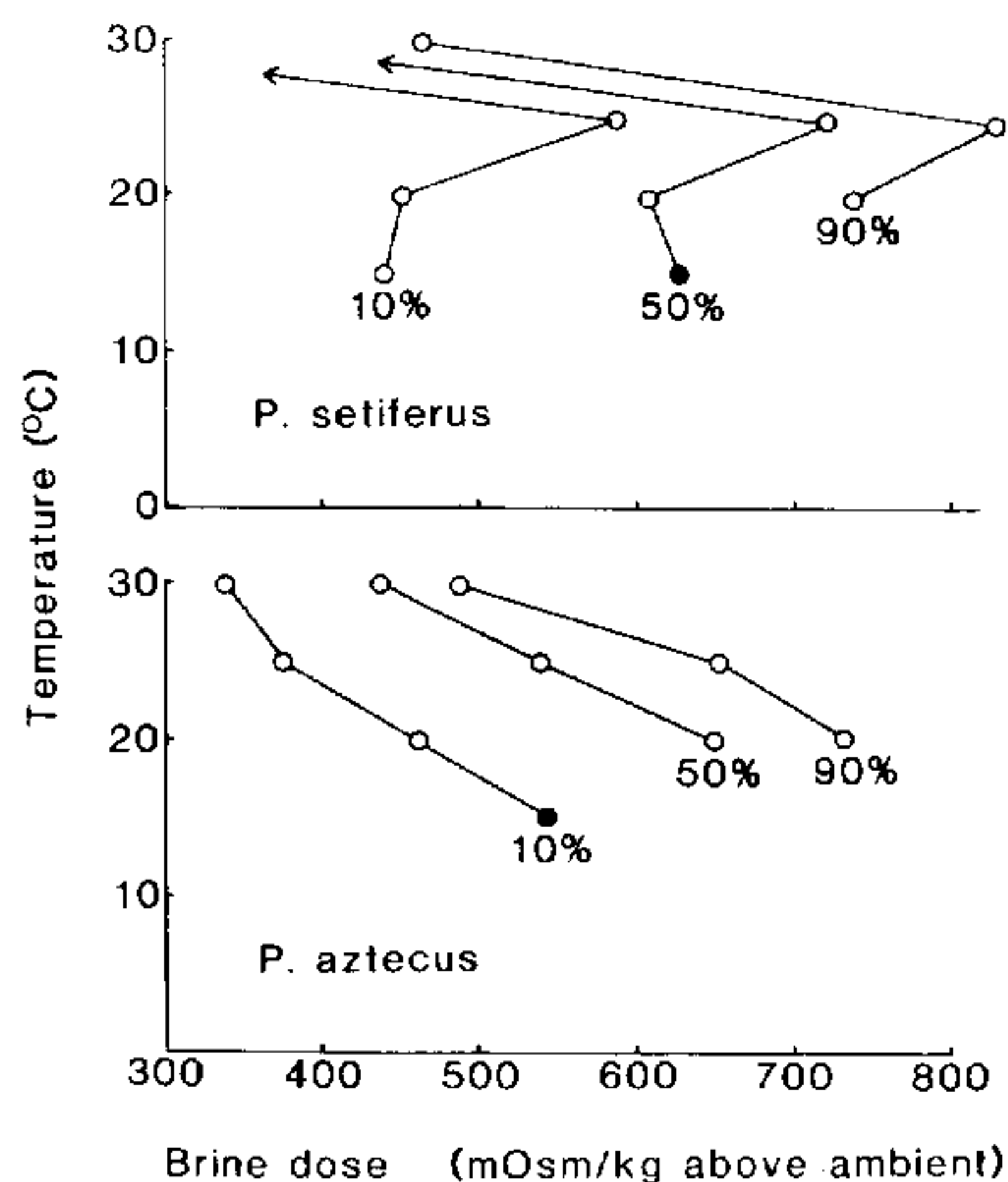


Fig. 3. Temperature-salinity response surfaces defined by three mortality levels. Closed circles are extrapolations

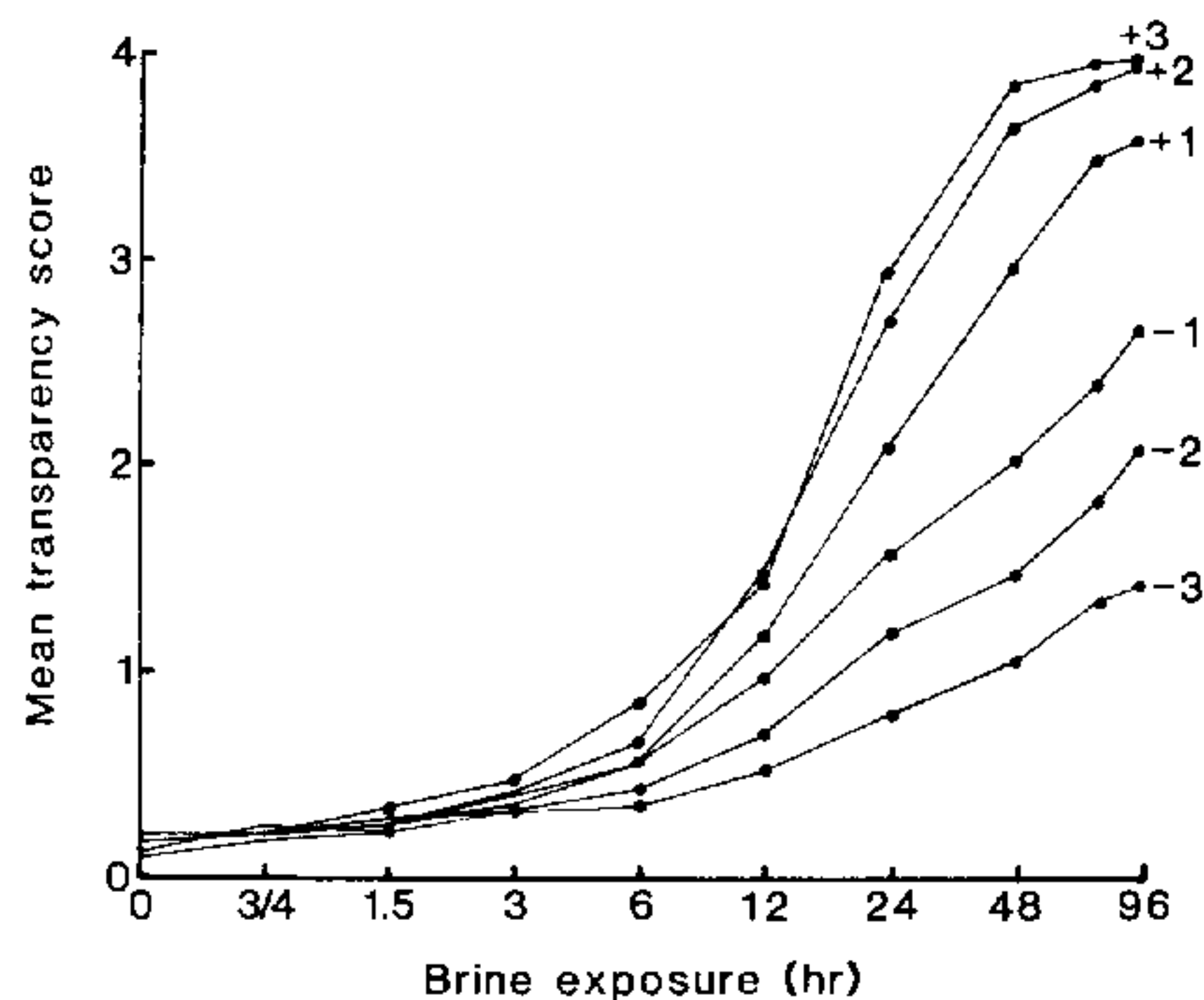


Fig. 4. Mean transparency score for all treatments as a function of duration of exposure to brine. Positive numbers at right identify doses progressively higher than LC50-96, and negative numbers identify progressively lower doses

mately 60% concordance in replicate measures by different observers. To make dose-responses comparable among experiments, doses were aligned with the LC50-96 for each experiment. The lowest dose that was higher than the LC50 was assigned a score of +1, and higher doses were given progressively higher scores. Similarly, doses lower than the LC50 were assigned negative scores. Since adjacent doses differed in osmolality by a factor of 1.17 in all experiments, the difference between doses scored -1 and -2 (for example) should have been comparable across experiments. Mean transparency at each observation time was averaged within an experiment, then across all 16 experiments. While individuals at doses well below LC50-96 became noticeably more opaque during the course of the experiments, shrimp exposed to

higher doses became markedly more opaque than those at low doses as early as 6 h after the beginning of the experiments, even before brine doses had reached their final values (Fig. 4).

Results with heart beat period (=interval between successive beats) were similar. For each group of five readings at each dose the median value was selected. Doses in each experiment were then divided into those above and those below LC50-96 for that experiment. Median values were then averaged within the high and low groups of doses in each experiment for each observation period, then across experiments for each species. By 6 h for *Penaeus setiferus* and by 12 h for *P. aztecus* the hearts of individuals at high brine doses beat significantly faster than those at low doses (Fig. 5). In both species heart beat period dropped at the 12-h observation, more noticeably for *P. aztecus* than for *P. setiferus*. This drop probably reflects the fact that the 12-h reading was the only reading made during the dark period. *P. aztecus* is a nocturnally active species, and individuals of *P. setiferus*, though usually more active during the day, were disturbed by the lights that were turned on for observations.

Of all dependent variables, behavior scores provided the earliest evidence for brine effects. Frequencies of scores of "moribund", "active," and "quiescent" were analyzed independently in two series of tests. As with heart rate (see above) doses were made comparable among experiments by pooling score frequencies for doses above and below LC50-96 for each experiment to form two groups at high and low doses, respectively. For the first analysis, score frequencies for the first five observation periods and the second five periods were separately

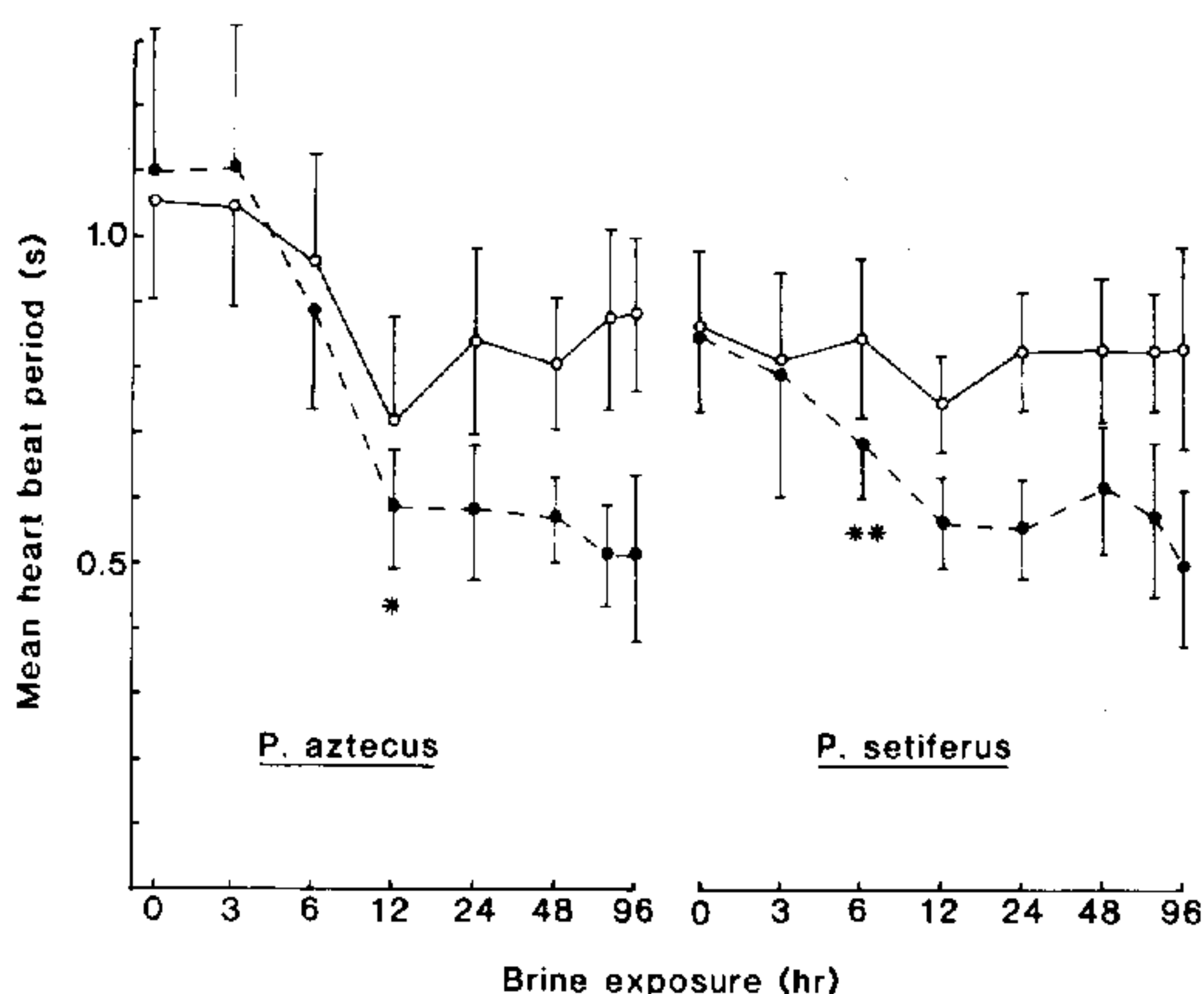


Fig. 5. Means for all treatment groups of the median heart beat period (time in seconds between beats) recorded for doses higher (solid symbols) or lower (open symbols) than LC50-96 as functions of duration of brine exposure for each species. Asterisks show the earliest times at which values for high and low doses differ significantly (* $P < 0.05$, ** $P < 0.01$). Vertical lines show 95% confidence intervals

pooled to make two groups, early and late. From this data set, 2×2 contingency tables were constructed to test for the independence of behavior score frequencies with salt type, diluent, season, species, dose and time of observation (Table 4).

Over all treatments, 2 653 (22.2%) of non-moribund scores were "active." Activity ("active" vs "quiescent") was significantly dependent (G test) on all other attributes except season. Compared with their respective alternatives, salt dome salt, Brazos River water, and high brine doses were associated with small increases (3.1 to 3.9%) in the frequency of "active", differences that were significant ($P < 0.01$) only because of the large number (11 968) of total observations. Species and time differences, on the other hand, were of large magnitude. White shrimp were active on 30.5% of 5 858 observations, whereas brown shrimp were active on only 14.2% of 6 110 observations. Individuals of both species were active on 33.1% of early observations but on only 18.2% of late observations. With equivalent brine doses, *Penaeus setiferus* was more active than *P. aztecus* at every observation period ($P < 0.05$), and the effect of high brine dose is an initial stimulation of activity (significant for *P. setiferus* at 0.75 h and for both species at 1.5 h), followed by a marked depression of activity (significant at 12 h for both species and at 24 h for *P. setiferus*) (Fig. 6).

The frequencies of "moribund" and live ("active" + "quiescent") observations were similarly tested for independence from the same set of experimental variables (Table 4). Significant non-independence occurred for salt

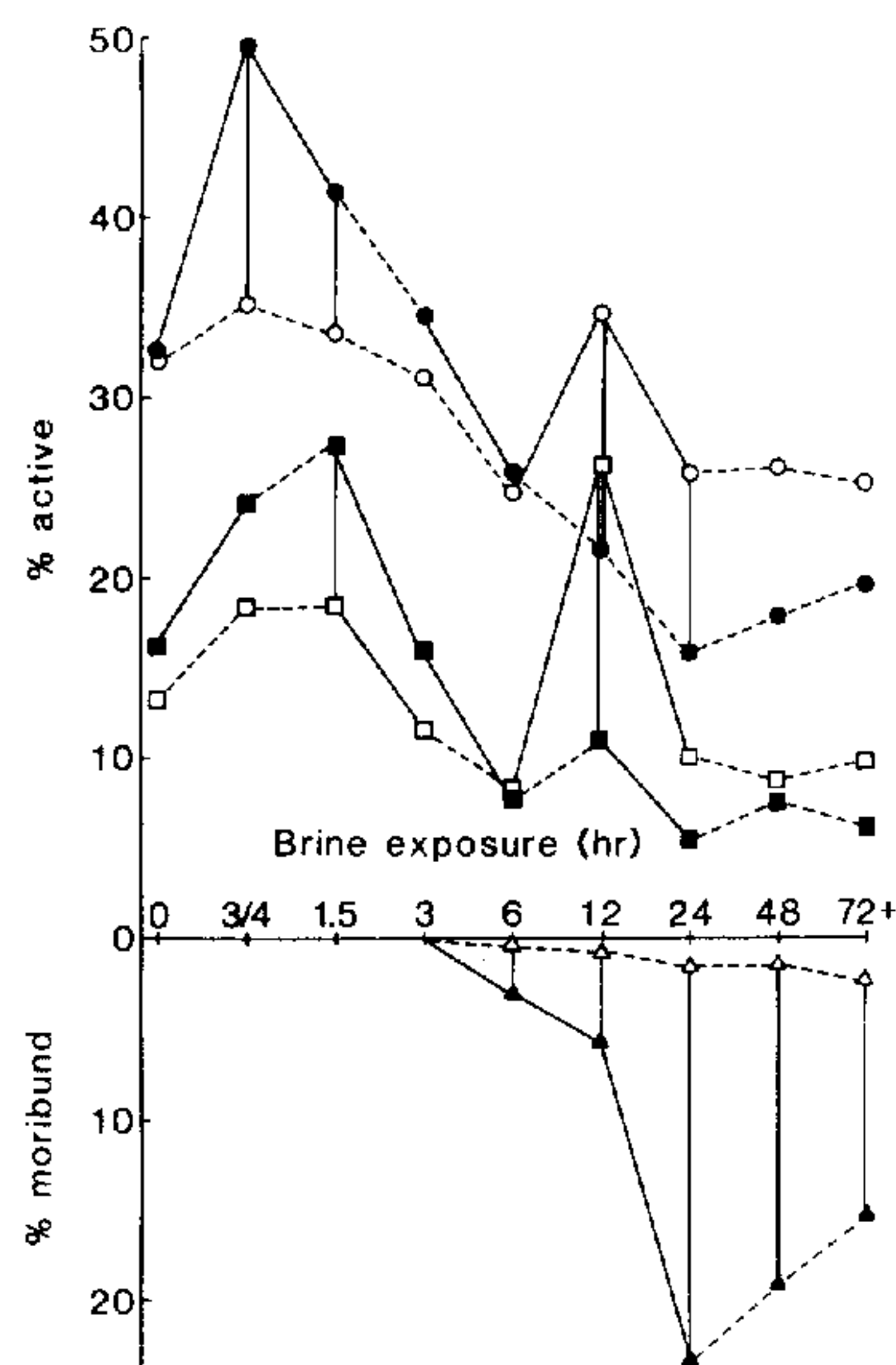


Fig. 6. *Penaeus setiferus* and *P. aztecus*. Active shrimp as percentages of all non-moribund shrimp by species (upper traces) and moribund shrimp as percentages of all shrimp (lower traces) as functions of duration of exposure to brine. Circles denote *P. setiferus*, squares, *P. aztecus*, and triangles, pooled species. Solid symbols denote values for doses above LC50-96; open symbols show lower doses. Solid lines connect consecutive or coincident proportions that differ significantly.

Table 4. Behavior score totals, summed over the two states of each of 6 test variables, with tests for independence. Minimum values of G causing rejection of independence are 3.84 ($P < 0.05$) and 6.63 ($P < 0.01$)

Variable	State	Active	Quiescent	G ^a	Moribund	G ^b
Salt type	Instant Ocean	1 301	5 019	19.4	117	36.6
	Salt dome	1 352	4 296		202	
	Deionized water	1 222	4 698		146	
Diluent	River water	1 431	4 617	15.8	173	1.7
	Fall	1 324	4 766		186	
Season	Spring	1 329	4 549	1.3	133	6.9
	<i>P. setiferus</i>	1 787	4 071		171	
Species	<i>P. aztecus</i>	866	5 244	469.3	148	2.7
	Low	1 553	5 814		67	
Dose level	High	1 100	3 501	13.0	252	213.2
	0–6 hr	1 771	5 346		28	
Time	12–96 hr	882	3 969	76.4	291	356.1

^a Tests active and quiescent totals versus other parameters

^b Tests moribund and sum of active and quiescent totals versus other parameters

type, season, dose, and time of observation. "Moribund" observations were about twice as frequent in salt dome brines (3.45%) as in synthetic sea salt brines (1.82%) and slightly more frequent in fall (2.96%) than in spring runs (2.21%). Only two shrimp, after being scored "moribund", ever recovered to receive "active" or "quiescent" scores; most died or stayed moribund until the end of experiments. It is not surprising, therefore, that dose and time of observation had marked effects on "moribund" frequency. Moribund shrimp were much more frequent at high brine doses (5.19%) than at low doses (0.90%) and in late observations (5.66%) than in early observations (0.39%). Frequencies of moribund shrimp at low and high brine doses differed by as early as 6 h after the initiation of brine flow (Fig. 6).

Individuals exposed to high experimental osmolalities did not osmoregulate well. Controls and brine-treated individuals were analyzed separately because departure from regression line was significantly smaller for controls than for experimentals ($F=34$, $P<0.01$) and because the slope of the control regression, 0.40, was significantly lower than 0.74, the slope of the experimental regression ($t'=2.73$, $P\approx 0.01$). The control regression predicts isosmoticity at 715 ± 8 (SE) mOsm kg^{-1} . Though results with individuals exposed to brine were more variable and the regression slope steeper, that slope was still significantly below 1 ($t=2.36$, $P\approx 0.025$), indicating some capacity for hyposmotic regulation over the range tested (Fig. 7).

Though heterogeneous variances between control and experimentals failed to satisfy assumptions for an analysis

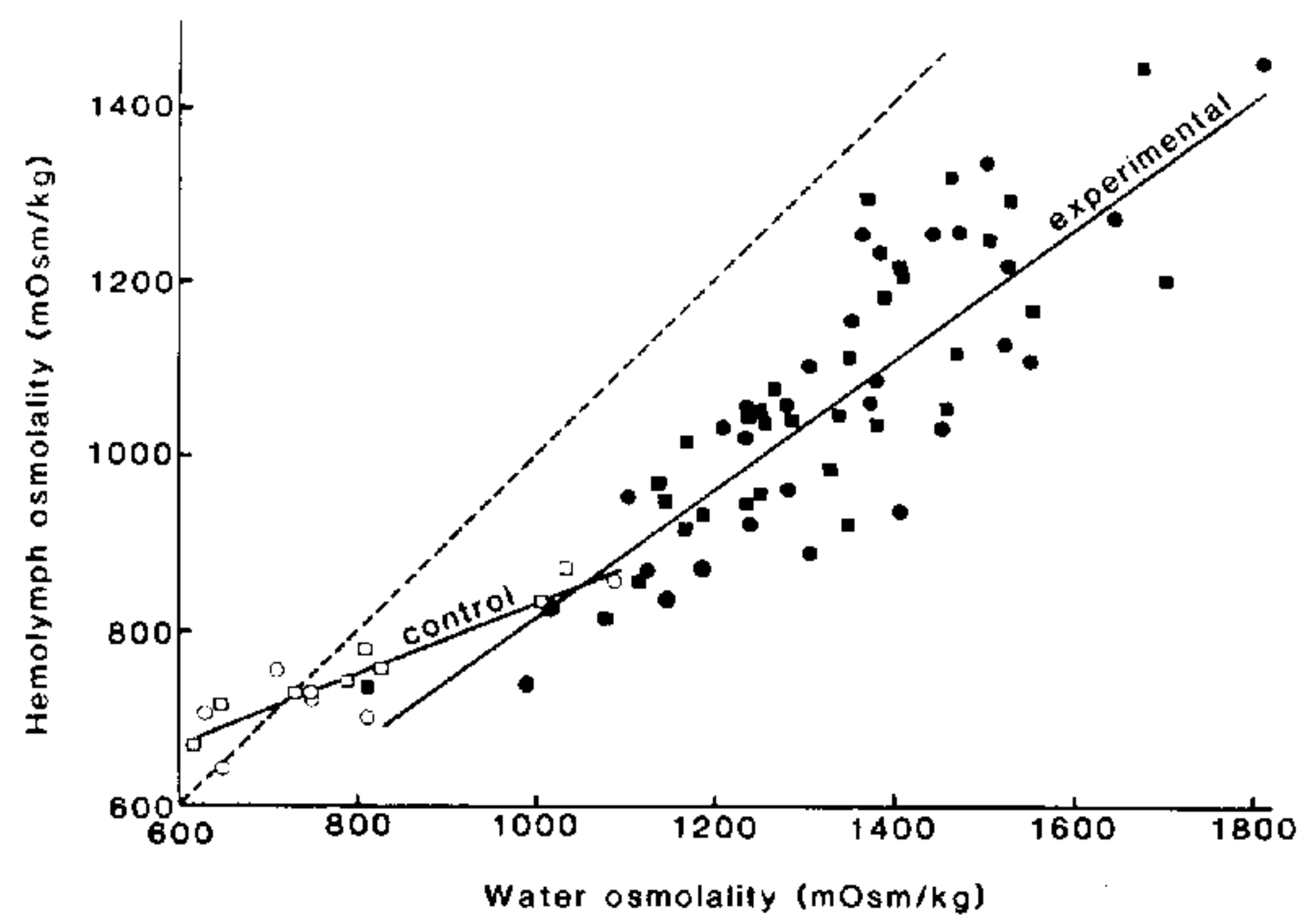


Fig. 7. Mean hemolymph osmolality for each treatment group as a function of tank osmolality. Solid lines were fitted separately to controls and brine-exposed shrimp. The dashed line is the isosmotic line

of variance, controls contributed so little (80 of 330 total determinations) to overall variance that analysis of covariance was performed on the entire data set in order to detect potential differences in osmoregulation caused by treatments. There was no significant difference between species in hemolymph osmolality (adjusted for covariance with environmental osmolality), but salt type, diluent type and season each had significant main effects and interactions (Table 5). There were tendencies for fall adjusted

Table 5. Hemolymph osmolality means (mOsm kg^{-1}) adjusted for covariance with environmental osmolality

Species	Instant Ocean				Salt Dome			
	Deionized water		River water		Deionized water		River water	
	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
<i>P. setiferus</i>	926	877	1 051	892	944	984	1 019	980
<i>P. aztecus</i>	981	951	1 012	922	967	993	1 022	938
Analysis of variance Source	Degrees of freedom				Mean square		F	
Species	1				13 063		1.72	
Salt	1				47 070		6.21 ^a	
Diluent	1				48 091		6.34 ^a	
Season	1				155 937		20.56 ^b	
Species \times salt	1				17 409		2.29	
Species \times diluent	1				45 102		5.95 ^a	
Salt \times diluent	1				5 265		0.69	
Species \times season	1				911		0.12	
Salt \times season	1				68 031		8.97 ^b	
Diluent \times season	1				136 295		17.97 ^b	
Covariance, environ. osmol.	1				7 000 789		922.94 ^b	
Error	318				7 785			

^a F statistics with significant sources of variance, $P<0.05$

^b F statistics with significant sources of variance, $P<0.01$

means to be higher than those for spring ($\bar{D}=48$ mOsm kg^{-1} , $t=2.1$, $df=7$, $0.05 < P < 0.10$) and for differences between fall and spring runs to be smaller for salt dome salt than instant ocean ($t=2.72$, 3 df , $0.05 < P < 0.10$). Differences between fall and spring runs were significantly larger for river water than for deionized water ($t=7.2$, 3 df , $P < 0.01$). Though the appropriate variance contributions were significant, and salt dome salt and Brazos River water brines evoked slightly higher body osmolalities, adjusted means did not differ according to salt type or water type by paired Student's *t*-tests.

Discussion

Under the conditions of these experiments, half of any group of adult and subadult individuals of *Penaeus setiferus* and *P. aztecus* could be expected to die when exposed for 48 h to water 654 ± 42 (95% CI) mOsm kg^{-1} above the previous environmental osmolality or for 96 h at 540 ± 41 (95% CI) mOsm kg^{-1} above normal. These values correspond to transfers from normal seawater (1 000 mOsm kg^{-1}) to water of 165 and 154% seawater salinities, respectively, or to salinities of approximately 56 and 52‰ S. Though it is not surprising that LC50-96 values are lower than those for LC50-48, it is possible that the difference represents a synergism between increased duration of exposure to high salinity and such experimental rigors as close confinement and starvation. Control survival was uniformly high (≤ 1 death in 15), but mere survival may not have guaranteed the maintained ability to withstand salinity stress.

A logarithmic series of 10 observations during toxicity experiments permitted estimates of mean lethal time as a function of brine dose. For both species LT50 appears to increase from about 20 h at 900 mOsm kg^{-1} above ambient to more than 200 h at 400 mOsm kg^{-1} above ambient. Estimates beyond this range were not practical, above 200 h because of the brevity of experiments and below 20 h because of the slow onset of brine exposure. Because of the internal consistency of LT50 estimates and their high sensitivities to dose, LT50 was used as the parameter to investigate potential effects of animal size on survival. In contrast to the finding of Venkataramiah *et al.* (1974) that sensitivity to elevated salinity increases with increasing size in smaller juvenile *Penaeus aztecus*, there was no evidence of increasing sensitivity with increasing size in adults and subadults of either species tested.

Neither LT50 or LC50 appeared to differ according to salt type, diluent type or season. This finding suggests that the major toxic effect measured in all experiments was osmotic stress. If Brazos River water had contained toxic constituents with measureable effects on mortality at the doses administered (approximately 10% brine in seawater at the highest doses) then experiments with Brazos River brine should have resulted in relatively low LT50's and LC50's. Likewise, if seawater-salt dome brine mixtures were toxic by virtue of altered ion ratios then one might have expected lower LC50's and LT50's in these experi-

ments using Instant Ocean brines, the ion ratios of which would have more closely approximated those in seawater. Finally, that season did not have a significant effect would suggest that long-term changes in the mortality of large shrimp exposed to high salinity were not of large magnitude compared with experimental error.

In the relationship of LT50 to dose (Fig. 1) there is no indication of a marked increase in slope with decreasing dose that would accompany a lethal threshold (Sprague, 1969), that is, a maximum dose below which no detectable effect on mortality occurred. It is therefore possible to extrapolate that relationship to worst-case predictions for salinity increases caused by brine discharge at the disposal site. If a 6.5‰ S increase is a worst case estimate based on one volume of saturated brine diluted in 45 volumes of seawater (NOAA, 1977), then the corresponding increase in osmolality should produce an LT50 of 1 275 h with a lower 95% confidence limit of 558 h for *Penaeus aztecus*, the species that provides the lower of the two estimates. Even if there is indeed no lethal threshold for brine, and shrimp were confined in the worst case exposure for as long as 558 h, both extremely unlikely events, it is probable that physiological adaptation to increased salinity would occur during so long an exposure, especially since 6.5‰ S above normal seawater salinity is well within the range of normal environmental salinities for both species (Simmons, 1957). The actual impact of brine may be still less severe. To date the highest salinity increase recorded at the diffuser site is 5‰ S for an area of 0.02 km^2 near the bottom directly over the diffuser (Randall, 1981). The evidence for temperature-salinity interactions presented here suggests that temperatures above 25 °C for both species and below 25 °C for *P. setiferus* should increase sensitivity to brine. These data clearly do not establish those temperature effects with much certainty due to small sample sizes and a limited dose series at temperatures of other than 25 °C. Even so, the data for *P. aztecus*, the principal species near the diffuser, suggest that the yearly maximum bottom temperature of 30 °C (Metzbower *et al.*, 1980) would be associated with 10% mortality at 48 h, providing the increased salinity reached 340 mOsm kg^{-1} (Fig. 3) or about 12‰ S, nearly twice the worst-case prediction for the near field, and six times the maximum 24-h exposure predicted in the NOAA (1978) model of brine plume behavior.

Results with sublethal parameters suggest that a rise in heart rate may be a good candidate for an indicator of incipient lethality due to osmotic stress, since significant dose-related differences in heart rate appear as soon as 6 and 12 h after the initiation of brine infusion, approximately as soon as the full-strength dose was established in these experiments. On the other hand, heart rate was extremely variable in these experiments. Heart rate was clearly affected by general level of activity and doubtless by many unmeasured and uncontrolled biological and environmental parameters. Tachycardia in response to salinity change is well documented in other crustaceans (DeFur and Mangum, 1979; Spaargaren, 1973, 1974). Dif-

ferences due to brine dose could only be detected by comparing the mean heart beat periods of groups of approximately 160 shrimp at frequent intervals, a requirement that may reduce the utility of bioassays based on heart beat.

Transparency was likewise an early indicator of osmotic stress in these experiments (Fig. 4). It suffers the same defect as heart rate for bioassays (i.e., low signal to noise ratio), and in addition suffers a defect that may limit its repeatability. In these experiments, observers of transparency could not have been blind to the treatment regime, and it is therefore possible that expectations of a dose effect could have influenced their judgements.

Short-term behavioral observations appear to offer a reasonably sensitive and consistent predictor of brine toxicity. Activity in both species was stimulated by toxic brine doses within about 45 min of the initiation of brine flow. At about 6 h, toxic brine concentrations began to depress activity (relative to quiescence), a depression that was most pronounced at 12 h, the only observation made during the night. *Penaeus aztecus*, a nocturnal species, was less active than *P. setiferus* during the day. Brown shrimp exposed to low brine doses became markedly more active at night, whereas those exposed to high doses did not significantly increase activity. It is noteworthy that the initial stimulation of activity evoked by brine occurred at a time when, at an exchange rate of 5 tank volumes per day, average brine concentrations in the tanks would have reached no more than 25% of the eventual values (Sprague, 1969). This suggests that brine-induced hyperactivity may be a response either to undiluted high-salinity water as it flows into test tanks or to high rates of salinity change but is probably not a response to high instantaneous salinity. An increased frequency of failure to orient properly ("moribund") distinguished shrimp at high and low brine doses after 6 h of brine flow, a time when dose levels had reached from 50–75% of the eventual values and when heart rate had also begun to respond (*P. setiferus*, Fig. 5).

Over the range of environmental osmolalities from 600 to 1 200 mOsm kg⁻¹, our measurements of hemolymph osmolality agree well with those reported for adult *Penaeus setiferus* (Castille and Lawrence, 1981). At their highest test osmolality, 1 160 mOsm kg⁻¹, the latter authors report mean hemolymph values of 900±18 (SE) mOsm kg⁻¹, compared with predictions for both species from regressions (Fig. 7) of 894±19 (SE) and 926±27 (SE) mOsm kg⁻¹ for controls and experimentals, respectively, at the same environmental osmolality. Our estimate of the isosmotic crossover point for both species, 715±8 (SE) mOsm kg⁻¹ lies between those reported for juvenile *P. aztecus* (745 mOsm kg⁻¹) and *P. setiferus* (680 mOsm kg⁻¹) by Castille and Lawrence (1981), but is well below other earlier estimates for large penaeids (Williams, 1960; McFarland and Lee, 1963; Bursey and Lane, 1971). Those higher estimates were based on data from short-term (24–96 h) exposures to salinity changes, whereas individuals used to generate our estimates (those not exposed

to brine) were continually exposed to test salinities for at least 10 d.

Many of our measurements were made on acutely-exposed shrimp that were either successfully acclimating or dying in response to high salinity. It is not surprising, therefore, that shrimp exposed to higher salinities regulated less well and displayed greater inter-individual variation than controls. Even so, our prediction of 1 350±19 (SE) mOsm kg⁻¹ for hemolymph in shrimp at an environmental osmolality of 1 750 mOsm kg⁻¹ lies within the range (1 125–1 450 mOsm kg⁻¹) reported by McFarland and Lee (1963) for *P. aztecus* gradually exposed to 1 750 mOsm kg⁻¹.

Though it was generally difficult to distinguish the effects of different brines or seasons on dose-related parameters, behavior and osmotic control provided exceptions. Evidence from those two approaches suggests that salt dome brines may provide a slightly more severe osmotic stress than synthetic sea salt brines. In salt dome brines osmotic regulation was somewhat poorer, especially in spring trials (Table 5), and the frequencies of moribund and active individuals were somewhat higher (Table 4). Both behavioral responses were also symptoms of high brine doses. Brazos River water brines appeared to be more stressful than deionized water brines by similar criteria: hemolymph osmolalities (especially in fall trials) and "active" frequencies were slightly higher for river water brines than for deionized water brines. Finally, fall trials were associated with significantly poorer osmoregulation and a higher incidence of moribund shrimp than spring trials.

The most attractive hypothesis to explain the differing effects of salt types is that the addition of salt mixtures with ion ratios markedly different from seawater, especially with respect to major divalent cations (Table 1), may make salt dome brines more stressful (Neff *et al.*, 1979). In the case of Brazos River water, it is possible that traces of toxicants from industrial (i.e., copper, Table 1) or agricultural (James, 1977) sources may intensify the effects of increased salinity. The seasonal effect may offer a simple explanation. Individuals for fall trials had generally experienced long prior exposure to temperatures lower than the test temperature of 25 °C. Results from the temperature-effect experiments (Fig. 4) suggest that cold-acclimated shrimp, brought to 25 °C over 24 or 48 h for tests, may have experienced a more severe osmotic challenge than those previously exposed to warmer temperatures.

None of these three hypotheses, however, are supported by the data on mortality: neither LT50 nor LC50 was sensitive to brine type or season. At least two factors could explain this apparent discrepancy. First, behavioral and osmotic measurements resulted in larger sample sizes than mortality observations. Mortality was recorded at only daily intervals during the period when most deaths occurred and could be recorded only once for each shrimp. Second, it is possible, though unlikely, that the behavioral parameters and reduced ability to osmoregulate were not strongly correlated with lethality.

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